PCT/EP 99 / 08980

Europäisches Patentamt | European Patent Office | Office européen des brevets

EP 99/ 8980

4

04 JAN 2000
PCT

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

98403015.5

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE, 27/12/99
LA HAYE, LE

Europäisches Patentamt
European Patent Office
Office eur péen des brevets

Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.: 98403015.5
Demande n°:

Anmeldetag:
Date of filing: 02/12/98
Date de dépôt:

Anmelder:
Applicant(s):
Demandeur(s):
I.D.M. IMMUNO-DESIGNED MOLECULES
75011 Paris
FRANCE

Bezeichnung der Erfindung:
Title of the invention:
Titre de l'invention:
New oligomeric conjugates liable to transfer biological molecules into cells

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

Staat: State: Pays:	Tag: Date: Date:	Aktenzeichen: File no. Numéro de dépôt:

Internationale Patentklassifikation:
International Patent classification:
Classification internationale des brevets:
C12N15/11, A61K47/48, A61K48/00

Am Anmeldetag benannte Vertragstaaten:
Contracting states designated at date of filing: AT/BE/CH/CY/DE/DK/ES/FI/FR/GB/GR/IE/IT/LI/LU/MC/NL/PT/SE
Etats contractants désignés lors du depôt:

Bemerkungen:
Remarks:
Remarques:

NEW OLIGOMERIC CONJUGATES LIABLE TO TRANSFER BIOLOGICAL MOLECULES INTO CELLS

The invention relates to new oligomeric conjugates liable to favor the transfer of biological molecules such as oligonucleotides, peptides and oligosides into cells.

The introduction of such molecules into cells is of great therapeutical interest.

Antisense oligonucleotides (ODN) and triplex forming oligonucleotides (TFO) are examples of attractive putative drugs in inhibiting or regulating gene expression in tumor cells and virus-infected cells.

Peptides from tumors and viruses are also attractive molecules in stimulating or elicitating a cell defense involving cytotoxic T lymphocytes against tumor and viruses after presentation by antigen presenting cells (APCs) such as dentrictic cells, macrophages and B cells.

But to be effective these molecules must reach their target in the right intracellular compartments which are either the cytosol or the nucleus.

Until now, studies of the intracellular location of oligonucleotides point out that in the majority of cells, most of the oligonucleotides are confined inside vesicles once taken up and only a small amount succeeds in reaching their RNA and DNA targets in the cytosol and in the cell nucleus.

Because once in the cytosol, the oligonucleotides penetrate rapidly into the nucleus, the enhancement of the delivery of oligonucleotides into the cytosol upon cell uptake, is expected to increase their biological activity.

Oligonucleotide encapsulation into liposomes increases their delivery into the cytosol but efficiency is drastically reduced in the presence of serum.

Antigenic peptides can bind to MHC Class I molecules and be presented at the cell surface of APCs, upon their processing via proteasomes located in the cytosol.

This has been achieved through the invention.

The invention, in one of its most general definitions, concerns a positively charged oligomeric conjugate containing an oligomer with a polymerization degree (PD) from 5 to 50, preferably 10 to 40 and more preferably 20, formed from monomeric components having free NH_3^+ in a number equal to or higher than 50 % of the polymerization degree,

said oligomer being as follows :

- the free NH_3^+ of the above-mentioned components are substituted in a ratio of at least 50 %, advantageously from 60 % to 95 %, particularly 80 to 90 % (this ratio being determined by nuclear magnetic resonance), by protonable residues in a weak acid medium, leading in such weak acid medium to a destabilization of cellular membranes,

- the above-mentioned protonable residues possess in addition the following properties :

→ they contain a functional group enabling them to be linked to the above-mentioned oligomer,

→ they do not correspond to a recognition signal recognized by a cellular membrane receptor,

→ they can comprise at least one free NH_3^+ group,

- the free NH_3^+ of the above-mentioned monomers can be also substituted by an uncharged residue leading to a reduction of the number of positive charges in comparison to the same oligomeric conjugate, before substitution,

- molecules constituting a recognition signal recognized by a membrane cellular receptor may be present :

→ either by substitution of some of the free NH_3^+ of the above-mentioned monomers,

→ either on some of the uncharged residues leading to a reduction of the number of charges,

→ either on some of the above-mentioned protonable residues leading to a destabilization of the cellular membranes,

the minimum amount of free NH_3^+ required, i.e. "at least" 10 free NH_3^+ come only from the α-NH_3^+ functions of the histidyl residues.

The destabilization of membranes means a modification of membranes which leads either to the increase of their permeability with respect to low molecular weight (and possibly high molecular weight) molecules in solution, or the fusion with another membrane.

The membrane permeability can be measured as follows :

Cells are incubated at 37°C for 30 min in DMEM medium without serum in the presence of 0.5 mg/ml fluorescein-labelled dextran (Mw 4000) and in the absence or in the presence of an oligomeric conjugate. Cells are then washed and incubated for 30 min at 37°C in culture medium containing 10% serum. Cells are fixed for 5 min in PBS containing 4 % paraformaldehyde and the cell fluorescence localization is analysed under a fluorescent confocal microscope.

The fusion of membrane can be measured as follows :

The fusion of membrane can be measured by using liposomes according to Struck et al., (Use of resonance energy transfer to membrane fusion. 1981 Biochemistry 20: 4093-4099).

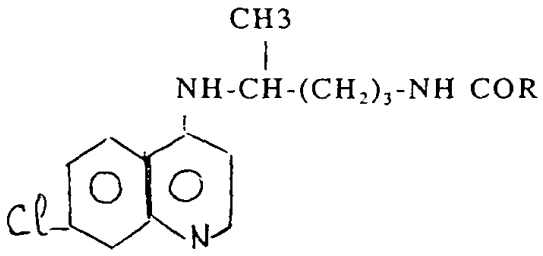
Dioleoylphosphatidylcholine (DOPC) liposomes containing N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) and octtadecylrhodamine (R18) as fluorescent energy transfer donor and acceptor lipid probes, respectively, are mixed with non fluorescent liposomes and incubated in the absence or in the presence of oligomeric conjugates at various pH. Membrane fusion is evidenced by a decrease of the rhodamine fluorescence emission at 585 nm upon excitation at 470 nm, as a consequence of a decrease of the resonance energy transfer between NBD and rhodamine when the average spatial separation induced by membrane fusion increases.

The residues accounting for the destabilization of cellular membranes act through their property of being protonable in a weak acid medium.

The expression "weak acid medium" designates a medium the pH of which is lower than that of plasma or serum, i.e. a pH lower than 7.4.

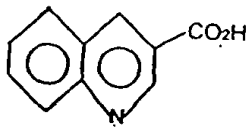
- they belong to the group of quinolins,
- they belong to the group of pterins,
- they belong to the group of pyridins.

An example of quinolin is represented by the following formulae :



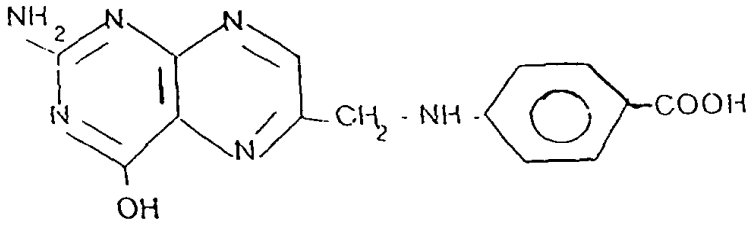
wherein R = $(CH_2)_nCO_2H$, in which n varies from 1 to 10, preferably from 1 to 3.

Another example of quinolin is :



(3-quinoline carboxylic acid)

An example of pterin is represented by the following formula :



(pteroic acid)

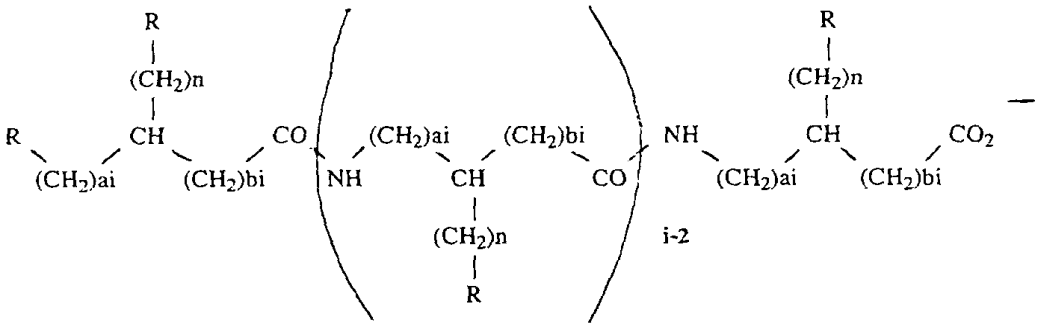
N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine,

8-(4-amino-1-methylbutylamino)-6-methoxy-quinoline (primaquine),

N^4-(6-methoxy-8-quinolinyl)1,4-pentanediamine, quininic acid,

quinoline carboxylic acid, pteroïc acid, nicotinic acid, quinolinic acid.

According to another advantageous embodiment, the oligomeric conjugate of the invention contains an oligomer of the following formula :



wherein

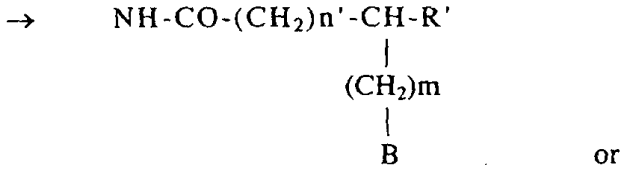
*ai is an integer varying from 0 to 10,

* bi is an integer varying from 0 to 10,

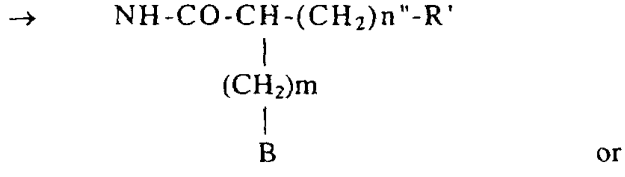
* i = degree of polymerization from 5 to 50, and particularly 10 to 40, and preferably 20,

* n = is an integer varying from 1 to 6, and preferably 4,

* R represents in a ratio of 50 % to 100 % (corresponding to a number u)

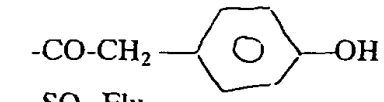


or



or

-CO-(CH_2)s-(CHOH)rH r being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 preferably 4

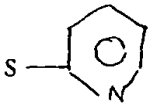


-SO_2-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

- H (corresponding to a number h)
- (CH_2) nH, n being an integer from 1 to 6 (corresponding to a number h)
- (CH_2) n-OH n being an integer from 1 to 6 (corresponding to a number h)
- (CH_2) n-SA' A' = H , CH_3 or 

n being integer from 1 to 6 (corresponding to a number h)

with
- i = u + j + k + h
- total number of α NH_3^+ = p = u-q
- total number of ω NH_3^+ = j = f-(k + h)
- total number of NH_3^+ = m = p + j + 1

with the proviso that :

1) u ≥ i/2
2) m ≥ i/2

According to another advantageous embodiment, the oligomeric conjugate of the invention contains an oligomer of the following formula :

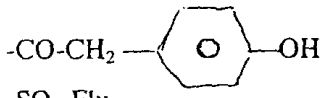
R' represents NH_3^+ (corresponding to a number p),

or NH (corresponding to a number q) substituted by

-CO-CH_3

-CO-(CHOH)rH — r being from 1 to 15 preferably 1 to 7

-CO-(CH_2)s-(CHOH)rH — r being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 and preferably 4



-SO_2-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

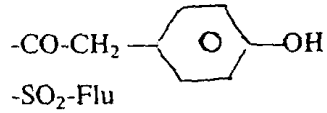
* R represents in a ratio of 0 % to 50 % (corresponding to f : $0 < f \leq 1$)

- NH_3^+ (corresponding to a number j),
- NH (corresponding to a number k), substituted by

-CO-CH_3

-CO-(CHOH)rH — r being from 1 to 15 preferably 1 to 7

-CO-(CH_2)s-(CHOH)rH — r and s being from 1 to 15 preferably 1 to 7 and s being from 1 to 6 and preferably 4



-SO_2-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

n' = n" = 0

R' = NH^+_3

m = 1

B = imidazole

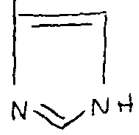
R = NH-CO-CH- NH_3^+

CH_2

(f) R = NH^+_3

u = 12

j = 7



or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'

(CH_2)m

B

wherein

n' = n" = 0

R' = NH^+_3

m = 1

B = imidazole

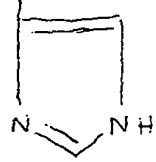
R = NH-CO-CH- NH_3^+

CH_2

(f) R = NH^+_3

u = 16

j = 3



or

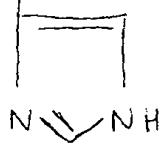
or

i = 19 n = 4 (l) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0
R' = NH^+_3
m = 1
B = imidazole
R = NH-CO-CH- NH_3^+
|
CH_2

(f) R = $CO-CH_3$
u = 15
k = 4



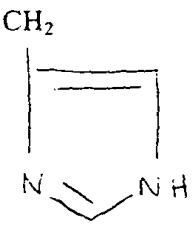
or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

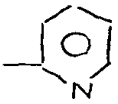
wherein

n' = n" = 0
R' = NH^+_3
m = 1
B = imidazole
R = NH-CO-CH- NH_3^+
|
CH_2

(f) R = CO-$(CHOH)_r$H
r = 5
u = 12
k = 3



n = 4 R' = H, CH_3, $CH(CH_3)_2$, CH_2,-$CH(CH_3)_2$
$CH(CH_3)$-CH_2-CH_3, CH_2OH,
CHOH- CH_3, CH_2-CH_2-S- CH_3
(CH_2)n H, (CH_2)n-OH, (CH_2)n-SA'

A' = H, CH_3 or 

R = NH-CO-(CH_2)n' -CH-(CH_2)n" -R"
|
(CH_2)m
|
B

t varies from 1 to 6

As an example, when, in the above formula n = 4, ai = bi = 0, t = 1, R' = H, the monomers are lysine and valine.

The invention also relates to a composition containing a mixture of at least one oligomeric conjugate as defined above, with at least one biological molecule, such as a peptide, an oligoside or an oligonucleotide derivative, or a mixture thereof.

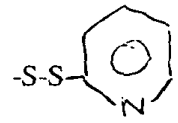
In the composition of the invention, the oligomeric conjugates can be associated with a biological molecule, in particular an oligoanion, such as an oligonucleotide, an anionic peptide or an anionic oligoside, via electrostatic interactions.

An anionic oligoside can be a sulfated oligoside, succinylated oligoside, phosphorylated oligoside, sialylated oligoside or an oligoside containing pyruvilidenyl groups.

The invention also relates to a combined preparation containing as active substance the following individual components, in the form of a kit-of-parts :

- at least an oligomeric conjugate as defined above,
- at least one biological molecule, such as a peptide, an oligoside or an oligonucleotide, or a mixture thereof,

each other H, OH, (CH_2)n-A, $[(CH_2)_2\text{-}O]_n\text{-}CH_2\text{-}CH_2\text{-}A$, A being H, OH, NH_2, COOH,



, n being an integer from 1 to 6,

E represents H, OH, OCH_3, OCH_2CH_3, $O(CH_2)_2CH_3$, $O(CH_2)_3CH_3$, $O(CH_2)_4CH_3$, $O\text{-}CH_2\text{-}CH_2\text{-}O\text{-}CH_3$

As example of oligonuclotides, one may cite the following :

GEM 91
phosphorothioate (X = S) oligonucleotide i = 25
CTC TCG CAC CCA TCT CTC TCC TTC T
complementary to the AUG initation site of gag HIV-1 gene

ISIS 1939
phosphorothioate (X = S) oligonucleotide i = 19
CCC CCA CCA CTT CCC CTC T
complementary to the 3' non coding region of ICAM-1 mRNA.

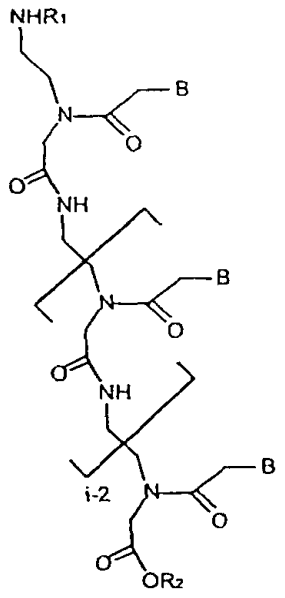
A mixture of an oligonucleotide and a peptide is defined as an oligonucleotide linked to a peptide, and a mixture of an oligonucleotide and an oligoside is defined as an oligonucleotide linked to an oligoside.

A mixture of an oligoside and a peptide is defined as an oligoside linked to a peptide.

A mixture of an oligonucleotide and a peptide or a mixture of an oligonucleotide and an oligoside used in the invention can have the following formulae :

When R1 and/or R2 represent a peptide, the mixed oligoanion is a peptido-oligonucleotide, and when R1 and/or R2 represent an oligoside, the mixed oligoanion represents a glyco-oligonucleotide.

An example of oligonucleotide is a peptide nucleic acid (PNA) represented by the following formula :



wherein - R1 and R2 represent independently from each other H, OH, $(CH_2)n$-A, $[(CH_2)_2$-$O]_n$-CH_2-CH_2-A, A being H, OH, NH_2, COOH,

- transfer into the cytosol and/or the cell nucleus of oligonucleotides corresponding to a repetitive bacterial type DNA sequence with stimulating or immunodepressive activity.

The transfer of an antisense oligonucleotide in the cytosol where it binds to the complementary mRNA sequence and blocks its traduction leading to inhibition of the synthesis of the gene product, can be carried out as described hereafter in the legends of Figures 1, 2 and 3.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of an oligonucleotide, wherein an oligonucleotide and an oligomeric conjugate as defined above, or of a composition as defined above, or of a combined preparation as defined above, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is :

- transfer into the cytosol and/or the cell nucleus of RNA or DNA oligonucleotide acting as decoys which inhibit gene expression by blocking the binding of regulatory factors to the authentic DNA region such as short RNA oligonucleotides corresponding to the HIV-TAR sequence inhibiting HIV expression and replication by blocking the binding of the HIV regulatory protein at Tat to the TAR region,

- transfer into the cytosol and/or the cell nucleus of ribozymes (RNA oligonucleotides) which inhibit gene expression by cleaving the mRNA.

The transfer into the nucleus of an oligonucleotide (triplex forming ODN, TFO) where it binds to target DNA at oligopurine sites where they form a triple-helical structure, leading to the inhibition of the gene expression, can be processed as hereafter described.

As an example of the specific TFO is an oligonucleotide 5'-$A_4GA_4G_6A$-3' directed against the polypurine track (PPT) in the NEF-HIV-1 gene.

The transfer of the oligonucleotide as activator of the immune response can be carried out as follows :

As an example the double strand RNA polyinosinic-polycytidylic acid (poly(I:C) for the increase of the tumoricidal activity of macrophages or for the stimulation of natural killer lymphocyte cytotoxicity.

A) Cells are incubated for 4 h at 37°C with 1 μM fluorescein-labelled peptide (*F-S*-CGEEDTSEKDEL) in the absence or in the presence of histidylated oligolysine. Cells are fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope.

B) Dendritic cells are incubated for 4 h at 37°C with 1 μM c-myc epitope peptide (SMEQKLISEEDLNFELDEA) in the absence or in the presence of histidylated oligolysine. Cells are fixed with 2 % of p-formaldehyde in the presence of 0.5 % saponine, washed and then incubated for 1 h with anti c-myc epitope monoclonal antibody (9E10) in PBS containing containing 10 mg/ml BSA and 0.1% saponin. Cells are washed and further incubated for 1 h in the presence of fluorescein-labelled anti-mouse IgG F(ab)' fragments in PBS containing containing 10 mg/ml BSA and 0.1% saponin. Cells are washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope.

The fixation of a peptide to intracellular cofactor can be carried as described in the following example:

An oligopeptide corresponding to a prostate specific antigen (PSA) epitope mixed to the oligomeric conjugate is transferred into the cytoplasm of macrophages.

The oligopeptide is fixed there to heat shock protein s (HSP90, HSP70) to form HSP–peptide complexes which are then re-expressed at the surface of macrophages. This complex formed with the HSP cofactor stimulate macrophages and enhance the immune response to the PSA antigen.

The invention also relates to a method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of peptide, wherein a peptide and an oligomeric conjugate as defined

containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a planapo objective (numerical aperture 1.4).

An example of transfer of a negatively charged oligoside can be illustrated by the following :

Cells are incubated for 4 h at 37°C with 0.5 mg/ml fluoresein-labelled polyanionic dextrans (either Mw 3000 or Mw 70000) in the absence or in the presence of an oligomeric conjugate. Cells are washed with PBS, fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a planapo objective (numerical aperture 1.4).

The fixation of an oligoside to intracellular cofactor can be carried as described in the following example:

An oligoanion with silicated saccharidic components complexed according to the invention is transported into the cytoplasm of human cells where it binds to intracellular cofactors or second messengers such as NF kappa B. This binding causes nuclear transfer of the cofactor which derepress or stimulates genes coding for cytokines (such IL1, TNF-α, IL-12).

This results in a marked stimulation of the human cell cultured in the presence of the complex.

The invention also relates to a pharmaceutical composition, comprising as active substance at least an oligomeric conjugate as defined above, or a composition as defined above, or a combined preparation as defined above, or in association with a pharmaceutically acceptable vehicle.

The invention also relates to the use of an oligomeric conjugate as defined above, or of a composition as defined above, or of a combined preparation as defined above, or for the preparation of a drug for use in the treatment of cancer, inflammatory or immunology diseases (such as graft rejection, allergy, auto-immunity) or infectious diseases.

37°C in DMEM supplemented with 2 % FBS containing various concentrations of GEM-91, (■) in the absence of histidylated oligolysine, ( ) in the presence of 20 μM HoK2 or (○) in the presence of 20 μM HoK3. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. Then, FBS was raised to 6 % and cells were further incubated for 18 h. Luciferase gene expression was measured by recording luminescence for 4 s. The percentage of luciferase inhibition was calculated by using , $[(RLU^{ODN}-RLU)/RLU] \times 100$ where RLU^{ODN} and RLU were the luciferase activity into cell lysates of cells incubated in the absence and in the presence of ODN, respectively. Results shown typical of experiments carried out in triplicate and repeated at least twice. Data are means ± standard deviation.

Inhibition of TNF-α induced ICAM-1 expression by ISI 1939.

Figure 2 shows the inhibitory effect of TNF-α induced ICAM-1 expression by ISIS 1939 (CCCCCACCACTTCCCCTCT), an antisense phosphorothioate oligonucleotide (PS-ODN) targeted to the 3' non-coding region of ICAM-1 mRNA. The results showed that TNF-α induced ICAM-1 expression was inhibited by ISIS 1939 in the presence of 20 μM of histidylated oligolysines. HoK2 (IC_{50} of 0.25 μM) appeared to be more efficient than HoK1 (IC_{50} of 0.5 μM) probably because HoK2 bore less histidyl residues than HoK1 (15 *versus* 12). The inhibition was very low in the absence of histidylated oligolysines even up to 1 μM ODN (20 % inhibition). A549 cells (ATCC CCL 185, ATCC Rockville, MD) were plated onto 96-wells microtiter plates (10^4 cells /well). The day after, culture medium was removed and cells were washed. Cells were incubated at 37°C for 4 h in 100 μl DMEM serum-free medium containing ISIS 1939 ODN either in the absence (■) or in the presence of 20 μM (●) HoK1 or (□) HoK2. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. One volume of fresh medium containing 10 ng/ml TNF-α was added and cells were further incubated for 18 h. ICAM-1 expression was quantified by ELISA using anti-ICAM-1 antibodies. Cells were washed 3 times with 200 μl of PBS and fixed for 20 min at room temperature in PBS containing 20 mg/ml paraformaldehyde. Then, cells were incubated for 90 min at 37°C with anti-ICAM 1 mouse antibody (Becton

ATCC Rockville, MD) were seeded onto sterile coverslips in 20-mm wells (2×10^5 cells/ well) and allowed to adhere. Cells were incubated in the presence of 0.125 μM fluorescein-labelled PS-ODN for 4 h at 37°C (a) in the absence or in the presence of 20 μM (b) HoK1, (c) HoK2, (d) HoK3 or (e) Plk (Oligolysine containing 19 lysyl residues and non substituted by hisditine used as control). Cells were fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells were analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope (Nikon, Tokyo, Japan) and a planapo objective (numerical apErture 1.4).

EXAMPLES

Preparation of histidylated oligolysines :

Oligolysine (Poly-L-lysine, HBr ; average molecular weight of 3950 ; average degree of polymerization of 19) (Bachem Feinchemikalien, Bubendorf, Switzerland) (1 g in 200 ml H_2O) was passed through an anion exchange column (Dowex 2 x 8, ^-OH form, 20-50 mesh) in order to remove bromide ions. The eluate was neutralized with a 10 % *p*-toluene sulfonic acid solution in water and freezed-dried.

Example 1 : Preparation of HoK1

Oligolysine *p*-toluene sulfonate salt (50 mg ; 8.6 μmol) in 2 ml dimethylsulfoxide (Aldrich, Strasbourg, France) in the presence of diisopropylethylamine (50 μl ; 344 μmol) (Aldrich) was reacted for 20 h at 20°C with (Boc)His(Boc)-OH (64 mg ; 146 μmol) (Novabiochem, Bad Soden, Germany) in the presence of benzotriazol-1-yl-oxy-tris-(dimethylamino) phosphonium hexafluorophosphate (BOP) (Richelieu Biotechnologies, Saint Hyacinthe, Canada) (159mg ; 358 μmol). The residual ε-amino groups of oligolysine was then substituted with gluconoyl residues (GlcA) : δ-gluconolactone (86mg ; 48 μmol)

H_2O/trifluoroacetic acid mixture (1 : 1 ; v/v) for 2 h at 20°C. Water and trifluoroacetic acid were removed under reduced pressure. HoK2 was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800 g for 15 min). The pellet was washed with isopropanol, collected by centrifugation (1800 g for 15 min), solubilized in distilled water and freezed-dried. The average number of histidyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy at 300 MHz in D_2O according to : $x = 6 . (h_{8.7} / h_{Lys})$. DP, where $h_{8.7}$ was the value of the integration of the signal at 8.7 ppm corresponding to the proton (1H C_{12}) of histidyl residues, h_{Lys} that in the range from 1.3 to 1.9 ppm corresponding to the 6 methylene protons (C_3, C_4 and C_5) of lysyl residues and DP the degree of polymerization of oligolysine. The number of histidyl residues bound per oligolysine molecule was 15. The average number of acetyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy from : $x = (h_A / h_{Lys})$. DP, where h_A was the value of the integration at 2.04 ppm of the 3 protons of acetyl residues, h_{Lys} that in the range of 1.3 to 1.9 ppm of the 6 methylene protons (C_3, C_4 and C_5) of lysyl residues and DP the degree of polymerization of pLK. The number of acetyl residues bound per oligolysine molecule was 3. The number of free ε-amino groups per oligolysinemolecule was 1.

Example 3 : Preparation of HoK3

Oligolysine *p*-toluene sulfonate salt (85 mg ; 14.6 μmol) in 3 ml dimethylsulfoxide in the presence of diisopropylethylamine (80 μl ; 555 μmol) was reacted for 20 h at 20°C with N-acetyl-His-OH (288 mg ; 307 μmol) (Sigma) in the presence of BOP (265 mg ; 597 μmol). The residual ε-amino groups of oligolysine was then acetylated (Ac) with acetic anhydride for 30 min at 20°C. HoK3 was precipitated by adding 10 volumes of isopropanol and spun down by centrifugation (1800 g for 15 min). The pellet was washed with isopropanol, collected by centrifugation (1800 g for 15 min), solubilized in distilled water and freezed-dried. The average number of histidyl residues bound per oligolysine molecule was determined by ^{1}H-NMR spectroscopy as describe The number of

The N-hydroxysuccinimidyl derivative of $(Boc)_2$-His-OH [$(Boc)_2$-His-OSu] (1g ; 2.2 mmol) is coupled to Fmoc-Lys-OH (722 mg ; 2.2 mmol) for 24 h at 20°C in dimethylformamide (ml). The Lys(His) synthon is precipitated with isopropanol, collected by centrifugation, washed with ether and dried under vaccum. The Lys(His) synthon is purified by cristallization.

Example 5 :

Preparation of an histidylated oligolysine (HoK) containing 20 lysine residues and 20 histidyl residues.

A HoK containing exactly 20 lysyl residues and 20 histidyl residues can be entirely synthetised by using the above Lys (His) synthon. Briefly, 20 Lys(His) synthon are successively assembled on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons are coupled by the HBTU activation method. HoK aree cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acis/water mixture (50% : 50% ; v/v) for 3 h at room temperature. Crude HoK is precipitated with isopropanol and collected by centrifugation. HoK is washed three times with isopropanol, resuspended in distilled water and freezed-dried.

Example 6

Preparation of an oligomeric conjugate containing 17 lysyl residues substituted with 17 histidyl residues and 3 leucyl residues inserted any where in the lys (His) sequence

Oligomers made of exactly 17 lysyl residues substituted with 17 histidyl residues and 3 leucyl residues insert anywhere in the Lys(His) sequence can be entirely synthetised by using the above Lys (His) synthon and Fmoc Leu on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons and Leu were coupled by the HBTU activation method. Oligomers are cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acis/water mixture (50% : 50% ; v/v) for 3 h at room temperature. Oligomers are precipitated with isopropanol and collected by centrifugation. Oligomers are washed three times with isopropanol, resuspended in distilled water and freezed-dried.

Table I : comparative evaluation of gene transfer and oligonucleotide transfer by using histidylated polylysine.

DP	His (%)	DNA	ODN	Cytotoxicity (%)
190	35	100	0	24
190	45	110	0	21
72	23	87-96	0	24
36	22	61-100	0	25
36	53	0-10	20	4
19	25	15-26	0	49
19	45	9	0	40
19	60	nd	100	26
19	80	nd	100	0
19	100	nd	100	0

DP is the oligolysine degree of polymerization. DNA corresponds to transfection by using histidylated oligolysine/pCMVLUC. The transfection efficiency is scored on a 0 to 100 scale. The transfection efficiency is determined from the luciferase activity in cells measured by luminescence. ODN corresponds to cytosolic and nuclear transfer of fluorescein-labelled oligonucleotide in the presence of histidylated oligolysine, evaluated under confocal microscope. Cytotoxicity was evaluated by using the colorimetric MTT assay. (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

CLAIMS

1. Oligomeric conjugate positively charged, containing an oligomer with a polymerization degree (PD) from 5 to 50, preferably 10 to 40 and more preferably 20, formed from monomeric components having free NH_3^+ in a number equal to or higher than 50 % of the polymerization degree,

said oligomer being as follows :

- the free NH_3^+ of the above-mentioned components are substituted in a ratio of at least 50 %, advantageously from 60 % to 95 %, particularly 80 to 90 % (this ratio being determined by nuclear magnetic resonance), by protonable residues in a weak acid medium, leading in such a weak acid medium to a destabilization of cellular membranes,

- the above-mentioned protonable residues possess in addition the following properties :

→ they contain a functional group enabling them to be linked to the above-mentioned oligomer,

→ they do not correspond to a recognition signal recognized by a cellular membrane receptor,

→ they can comprise at least one free NH_3^+ group,

- the free NH_3^+ of the above-mentioned monomers can be also substituted by an uncharged residues leading to a reduction of the number of positive charges in comparison to the same oligomeric before substitution,

- molecules constituting a recognition signal recognized by a membrane cellular receptor may be present :

→ either by substitution of some of the free NH_3^+ of the above-mentioned monomers,

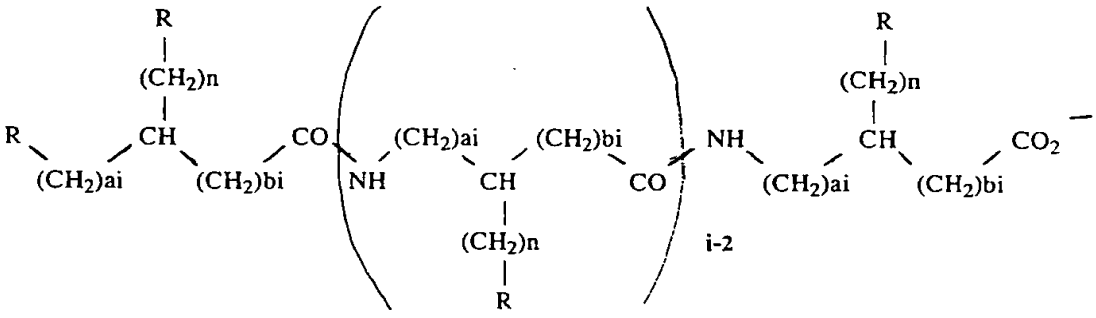
→ either on some of the uncharged residues leading to a reduction of the number of charges,

→ either on some of the above-mentioned protonable residues leading to a destabilization of the cellular membranes,

5. Oligomeric conjugate according to anyone of claims 1 to 4, wherein the protonable residues leading to a destabilization of cellular membranes are chosen from :

histidine, 4-carboxymethyl-imidazole,
3-(1-methyl-imidazol-4yl)-alanine, 3-(3-methyl-imidazol-4yl)-alanine,
2-carboxy-imidazole, histamine, 3-imidazol-4yl)-L-lactic acid,
2-(1-methyl-imidazol-4yl)ethylamine, 2-(3-metyl-limidazol-4yl)ethylamine,
β-alanyl-histidine-(carnosine), 7-chloro-4(amino-1-methylbutylamino)-quinoline,
N^4-(7-chloro-4-quinolinyl)-1,4-pentanediamine,
8-(4-amino-1-methylbutylamino)-6-methoxy-quinoline (primaquine),
N^4-(6-methoxy-8-quinolinyl)1,4-pentanediamine, quininic acid,
quinoline carboxylic acid, pteroïc acid, nicotinic acid, quinolinic acid.

6. Oligomeric conjugate according to anyone of claims 1 to 5, wherein the oligomeric conjugate contains an oligomer of the following formula :



wherein * ai is an integer varying from 0 to 10,

* bi is an integer varying from 0 to 10,

* i = degree of polymerization from 5 to 50, and particularly 10 to 40, and preferably 20,

* n = is an integer varying from 1 to 6, and preferably 4,

* R represents in a ratio of 50 % to 100 % (corresponding to a number u)

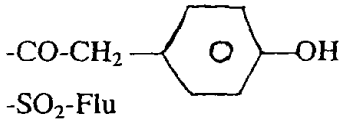
* R represents in a ratio of 0 % to 50 % (corresponding to f : $0 < f \leq u$)

- NH_3^+ (corresponding to a number j),
- NH (corresponding to a number k), substituted by

$-CO-CH_3$

$-CO-(CHOH)rH$ r being an integer from 1 to 15, and preferably 1 to 7

$-CO-(CH_2)s-(CHOH)rH$ r being an integer from 1 to 15,and preferably 1 to 7, and s being an integer from 1 to 6, and preferably 6



$-SO_2$-Flu

-CO-Flu

-CS-NH-Flu

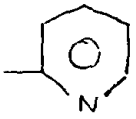
Flu being a fluorescent molecule

- H (corresponding to a number h)
- (CH_2) nH, n being an integer from 1 to 6

(corresponding to a number h)

- (CH_2) n-OH n being an integer from 1 to 6

(corresponding to a number h)

- (CH_2) n-SA' A' = H, CH_3 or 

(corresponding to a number h) n being integer from 1 to 6

with
- $i = u + j + k + h$
- total number of α $NH_3^+ = p = u-q$
- total number of ω $NH_3^+ = j = f-(k + h)$
- total number of $NH_3^+ = m = p + j+1$

→ $NH\text{-}CO\text{-}(CH_2)n'\text{-}CH(B)\text{-}(CH_2)m\text{-}(CH_2)n''\text{-}R'$

m is an integer varying from 1 to 6,

n' is an integer varying from 0 to 6,

n" is an integer varying from 0 to 6,

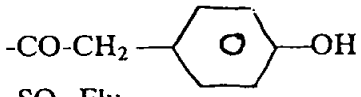
B is a weak base as defined according to anyone of claims 2 to 4,

R' represents NH_3^+ (corresponding to a number p),
or NH (corresponding to a number q) substituted by

$-CO-CH_3$

$-CO-(CHOH)rH$ r being an integer from 1 to 15, and preferably 1 to 7

$-CO-(CH_2)s-(CHOH)rH$ r being an integer from 1 to 15, and preferably 1 to 7, and s being an integer from 1 to 6, and preferably 6



$-SO_2$-Flu

-CO-Flu

-CS-NH-Flu

Flu being a fluorescent molecule

* R represents in a ratio of 0 % to 50 % (corresponding to $f : 0 < f \leq 1$)

- NH_3^+ (corresponding to a number j),
- NH (corresponding to a number k), substituted by

$-CO-CH_3$

$-CO-(CHOH)rH$ r being an integer from 1 to 15, and preferably 1 to 7

i = 19 n = 4 (u) R= NH-CO-$(CH_2)n'$-CH-$(CH_2)n''$-R'
|
$(CH_2)m$
|
B

wherein

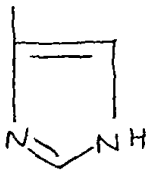
n' = n'' = 0

R' = NH^+_3

m = 1

B = imidazole

R = NH-CO-CH- NH_3^+
|
CH_2



(f) R = NH^+_3

u = 12

j = 7

or

i = 19 n = 4 (u) R= NH-CO-$(CH_2)n'$-CH-$(CH_2)n''$-R'
|
$(CH_2)m$
|
B

wherein

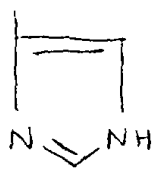
n' = n'' = 0

R' = NH^+_3

m = 1

B = imidazole

R = NH-CO-CH- NH_3^+
|
CH_2



(f) R = NH^+_3

u = 16

j = 3

or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0

R' = NH^+_3

m = 1

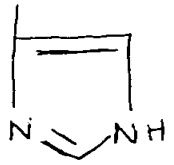
B = imidazole

R = NH-CO-CH- NH_3^+
|
CH_2

(f) R = $CO-CH_3$

u = 15

k = 4



or

i = 19 n = 4 (u) R= NH-CO-(CH_2)n' -CH-(CH_2)n"-R'
|
(CH_2)m
|
B

wherein

n' = n" = 0

R' = NH^+_3

m = 1

B = imidazole

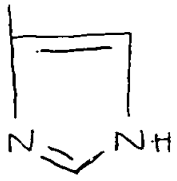
R = NH-CO-CH- NH_3^+
|
CH_2

(f) R = CO-(CHOH)rH

r = 5

u = 12

k = 3



11. Use of an oligomeric conjugate according to anyone of claims 1 to 8, for the *in vitro*, the *ex vivo* or the *in vivo* intracellular transfer of biological molecules into the cytosol and/or in the cell nucleus.

12. Use of an oligomeric conjugate according to anyone of claims 1 to 8 or of a composition according to claim 9, or of a combined preparation according to claim10, for the intracellular the *in vitro*, the *ex vivo* or the *in vivo* transfer of a peptide, an oligoside or an oligonucleotide, or a mixture thereof, into the cytosol or/and in the cell nucleus.

13. Use of an oligomeric conjugate according to anyone of claims 1 to 8 or of a composition according to claim 9, or of a combined preparation according to claim10, wherein the cells are chosen among muscular, epithelial, endothelial, myeloid cells such as monocytes, macrophages and fibroblasts, leukocytes and granulocytes, osteoblasts as well as dendritic cells, stem cells, neuronal cells, or dermal cells.

14. Method for the *in vivo*, the *in vitro* or the *ex vivo* transfer of an oligonucleotide, wherein an oligonucleotide and an oligomeric conjugate according to anyone of claims 1 to 8 or of a composition according to claim 9, or of a combined preparation according to claim10, are(is) contacted with a medium containing cells to be transferred, under conditions such that there is :

- transfer of an antisense oligonucleotide in the cytosol and/or the cell nucleus where it binds and blocks the complementary mRNA sequence,
- or transfer of an oligonucleotide as activator into the cytosol where it depresses or activates a second messenger in the cytosol, or the corresponding gene in the nucleus,
- or transfer into the cytosol and/or the cell nucleus of oligonucleotides corresponding to a repetitive bacterial type DNA sequence with stimulating or immunodepressive activity,

18. Use of an oligomeric conjugate according to anyone of claims 1 to 8, or of a composition according to claim 9, or of a combined preparation according to claim10, or for the preparation of a drug for use in the treatment of cancer, inflammatory or immunology diseases (such as graft rejection, allergy, auto-immunity) or infectious diseases.

19. Kit or case containing :

- an oligomeric conjugate according to anyone of claims 1 to 8, substituted by a protonable residue leading in a weak acid medium to a destabilization of cellular membranes, this oligomeric conjugate being able to comprise a recognition signal, which is previously fixed or not on the above-said conjugate, said recognition signal being dependent upon the cell to target,
- at least one biological molecule to transfer,
- optionally reagents enabling the possible binding of the recognition signal on the above-said oligomeric conjugate,
- optionally reagents enabling the formation of a composition according to claim 9, or of a combined preparation according to claim 10,
- reagents enabling the transfer of the biological molecule in the cytosol and/or the cell nucleus.

55

ABSTRACT

NEW OLIGOMERIC CONJUGATES LIABLE TO TRANSFER BIOLOGICAL MOLECULES INTO THE CELLS

The invention relates to a positively charged oligomeric conjugate, containing an oligomer with a polymerization degree (PD) from 5 to 50, preferably 10 to 40 and more preferably 20, formed from monomeric components having free NH_3^+ in a number equal to or higher than 50 % of the polymerization degree.

No figure.

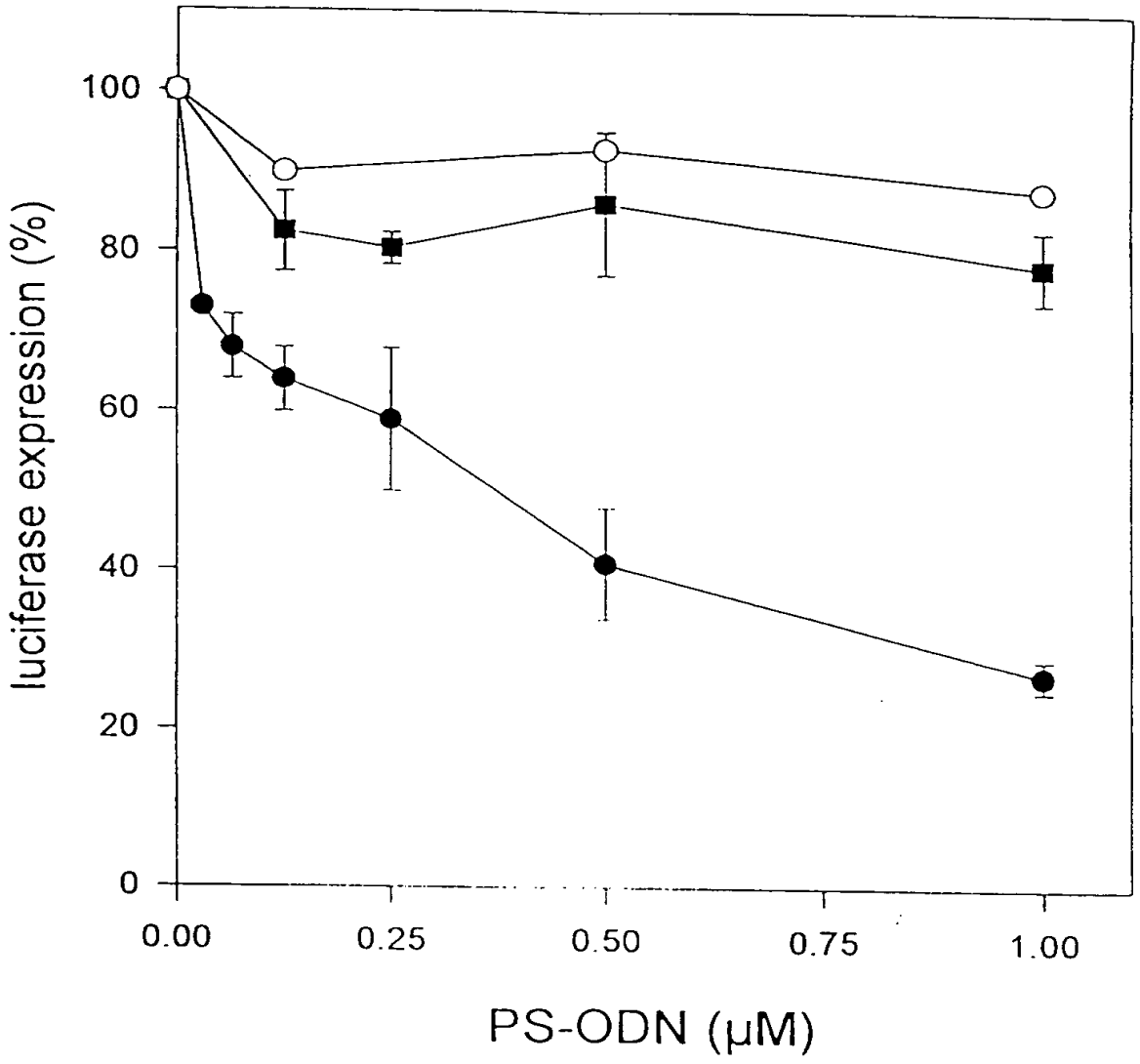

100
80
60
40
20
0
luciferase expression (%)
0.00
0.25
0.50
0.75
1.00
PS-ODN (μM)

Figure 1

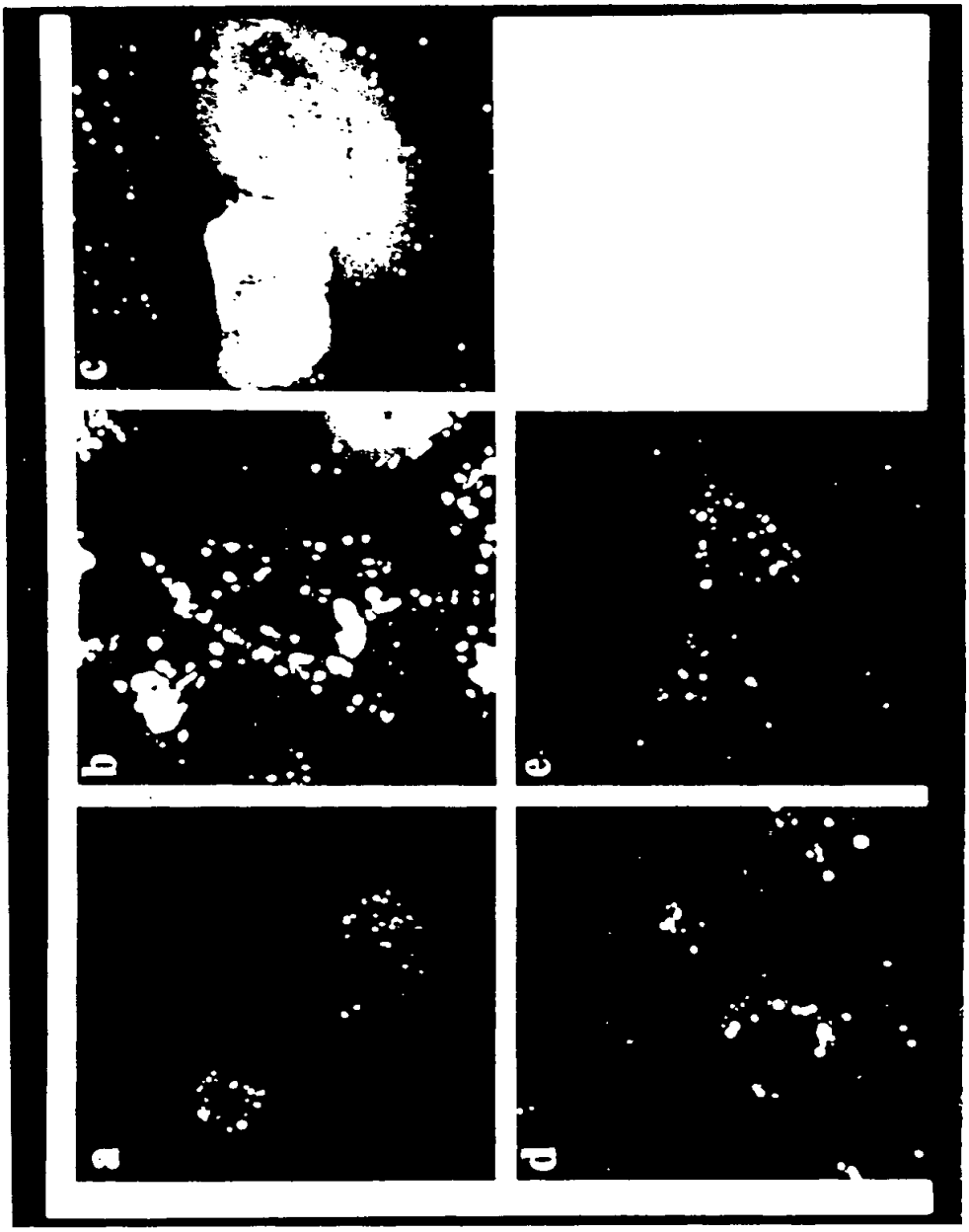


Figure 3